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FEATHER CORTICOSTERONE LEVELS AND CAROTENOID-BASED COLORATION IN COMMON BUZZARD (*BUTEO BUTEO*) NESTLINGS

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Abstract.—Most of our understanding of the function of colored traits displayed by birds and the mechanisms that produce or maintain them comes from studies on adults. However, colored traits are often displayed by nestlings from a young age, and these traits may influence parent-offspring interactions or sibling competition. The mechanisms that may mediate the expression of those traits during growth are still fairly unknown in raptors. In this study, we examined a possible mediating effect of corticosterone levels on the expression of carotenoid-pigmented traits in nestlings of Common Buzzards (*Buteo buteo*), specifically the yellow-orange coloration of their cere and legs. We assayed corticosterone levels deposited in feathers, which can provide a reliable and integrated index of stress responses during growth. Carotenoids can be used to color integuments, or diverted to other physiological processes involved in self-maintenance. We hypothesized that corticosterone levels mediate how carotenoids can be diverted to functions other than coloration. We show that carotenoid and corticosterone levels were positively associated, perhaps because of a higher metabolic activity in more-stressed nestlings. Corticosterone levels were negatively correlated with the coloration of cere and legs in females only. Altogether, our results support the hypothesis that corticosterone may influence how carotenoid pigments are allocated for needs other than coloration, although in a sex-specific manner. We encourage further studies exploring how individuals cope with and respond to stressful conditions, in order to better understand the complex interactions between corticosterone, carotenoids, and coloration during nestling growth.

Key Words: *Common Buzzard*; Buteo buteo; *feather corticosterone*; *growth*; *sexual dimorphism*; *stress response.*

NIVELES DE CORTICOSTERONA EN LAS PLUMAS Y COLORACIÓN BASADA EN CAROTENOIDES EN PICHONES DE *BUTEO BUTEO*

Resumen.—La mayoría de nuestro entendimiento de la función de los atributos coloreados desplegados por las aves y los mecanismos que los producen o mantienen vienen de estudios realizados en adultos. Sin embargo, los atributos coloreados son a menudo desplegados por los pichones desde una edad muy temprana, y estos rasgos pueden influenciar en la interacción padres - crías o en la competencia entre hermanos. Los mecanismos que pueden mediar la expresión de estos atributos durante el crecimiento son todavía bastante desconocidos en las rapaces. En este estudio examinamos un posible efecto de mediación de los niveles de corticosterona en la expresión de los atributos pigmentados por carotenoides en pichones de *Buteo buteo*, específicamente la coloración amarillo-anaranjada de sus ceras y patas. Valoramos los niveles

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de corticosterona depositados en las plumas, los cuales pueden servir como un índice confiable e integrado de respuesta al estrés durante el crecimiento. Los carotenoides pueden ser usados para colorear integumentos o desviados hacia otros procesos fisiológicos involucrados en el auto mantenimiento. Hipotetizamos que los niveles de corticosterona influyen en cómo los carotenoides pueden ser desviados hacia otras funciones aparte de la coloración. Demostramos que los niveles de carotenoides y corticosterona estuvieron positivamente asociados, quizá debido a una actividad metabólica mayor en los pichones más estresados. Los niveles de corticosterona estuvieron negativamente correlacionados con la coloración de la cera y las patas, pero sólo en las hembras. En conjunto, nuestros resultados apoyan la hipótesis de que la corticosterona puede influenciar en cómo los pigmentos carotenoides son distribuidos para otras necesidades además de la coloración, aunque de una manera específica en cada sexo. Alentamos la realización de estudios adicionales que exploren cómo los individuos se enfrentan y responden a situaciones estresantes, de manera de entender mejor las complejas interacciones entre la corticosterona, los carotenoides y la coloración durante el crecimiento de los pichones.

[Traducción del equipo editorial]

Animal communication signals can be defined as phenotypic traits produced by the signaler that have evolved because they change the behavior of receivers in ways that benefit the signaler (Maynard Smith and Harper 2003). Most of our understanding of these issues comes from the mechanisms that explain variation in the expression of sexual signals and its evolutionary role. Secondary sexual traits are expressed during adulthood, but young, dependent individuals also display conspicuous traits to their parents and siblings (Godfray 1995). The expression of colored traits in nestlings such as the gape, skin, or plumage have been associated with individual condition, health, or food needs (Kilner 1997, Saino et al. 2003, Bize et al. 2006, Galvan et al. 2008) and shown to play a role in sibling competition or parent-offspring communication (Saino et al. 2000, 2002, Strasser and Schwabl 2004, Tschirren et al. 2005, Fargallo et al. 2007, Loiseau et al. 2008, Parejo et al. 2010, Aviles and Parejo in press).

During growth, birds face multiple sources of stress such as food shortages, inclement weather, parasitism, and predation risk. The mechanism that regulates the response to stressors is the hypothalamic–pituitary–adrenal axis, which releases glucocorticoids to recover homeostasis (Wingfield et al. 1998). The capacity of individuals to cope with stress is essential for growth, survival, and reproduction (Romero and Wikelski 2001, Romero 2004, Blas et al. 2007, Romero and Wikelski 2010). However, chronic stress can have serious negative effects (Romero 2004), such as immunosuppression (Buchanan 2000). If the expression of phenotypic traits honestly indicates an individual's quality, then levels of trait expression should be influenced by stress levels in general, and should co-vary with stress hormone (glucocorticoid) levels (Bortolotti et al. 2009a). Reliably quantifying stress responses in animals using variations in blood corticosterone levels has always been a challenging task, because of short-term responses to perturbations, such as handling (Romero 2004). However, assessing corticosterone levels in feathers provides an integrative measure of stress responses (Bortolotti et al. 2008, 2009a). Feather corticosterone levels can be interpreted as a summarized index of the overall amount of corticosterone produced during feather growth, and therefore a proxy of stress levels of individuals during growth (Bortolotti et al. 2008, 2009a). How an individual responds to stressors may be an important determinant of its fitness, and it was recently suggested that ornaments and colored traits could reveal how individuals respond to stressors (Loiseau et al. 2008, Bortolotti et al. 2009b).

Carotenoid-pigmented traits are perhaps among the most common colored traits in birds, including raptors (Hill and McGraw 2006). In raptors, carotenoids provide the yellow-red coloration of features such as the cere, tarsi, and other bare parts (Table 1). In many raptor species, nestlings display the carotenoid-colored structures from a very early age (Table 1) and there is growing evidence that carotenoid-pigmented features reliably signal nestling condition or quality in raptors (Table 1). Moreover, there is recent experimental evidence that the coloration of bill or cere of raptor nestlings plays a role in parent-offspring communication and influences parental food-provisioning to nestlings (Parejo et al. 2010, Aviles and Parejo in press). However, the physiological mechanisms that influence the expression of these traits during growth are still relatively unknown, especially in raptors. Carotenoids have to be ingested, as birds cannot synthetize them *de novo*, and are powerful immunostimulants (Blount et al. 2003, McGraw and Ardia 2003). Once carotenoids are ingested, birds and other vertebrates bind carotenoids to lipoproteins in the blood, distributing lipids to peripheral

Table 1. Summary of studies exploring the function or cost of producing carotenoid-pigmented traits in nestling raptors. Note that other studies investigated variation of carotenoid levels in nestling American (Bortolotti et al. 2000) and Eurasian Kestrels (Costantini et al. 2007, Laaksonen et al. 2008, Neve et al. 2008), but not the association with coloration. In the column ''color assessed,'' the index of color that was used in each case is specified in parentheses.

SPECIES	TRAIT ASSESSED	EXPERIMENTAL	COLOR ASSESSED	PARAMETER OF INDIVIDUAL QUALITY MEASURED	DIRECTION OF THE CORRELATION	REFERENCES
Eurasian Kestrel (*Falco tinnunculus*)	Tarsus	Yes (carotenoid supplementation)	Yellowness (hue)	Body condition	Positive	Casagrande et al. 2007
	Tarsus	Yes (cross fostering)	Yellowness (hue)	None	N/A	Casagrande et al. 2009
Common Buzzard (*Buteo buteo*)	Cere/tarsus	No	Yellowness (hue, chroma and brightness)	Nestling mass and cholesterol	None	This study
Common Scops-Owl (*Otus scops*)	Cere	Yes (UV reflectance)	UV (brightness, chroma and λUV peak)	Nestling mass/food provisioning	Negative (higher UV reflectance, lower body mass gain)	Parejo et al. 2010
Little Owl (*Athene noctua*)	Cere/beak	Yes (yellowness reflectance)	Yellowness (brightness, yellow-red chroma, UV chroma)	Nestling mass/food provisioning	Positive	Aviles and Parejo in press
Montagu's Harrier (*Circus pygargus*)	Cere/tarsus	Yes (carotenoid supplementation)	Yellowness[1]	Body condition/ T-cell-mediated immunity	None/positive	Sternalski et al. 2010
	Cere/tarsus	Yes (carotenoid supplementation)	Yellowness[1]	Body condition/T-cell-mediated immunity	None/positive (interaction with nestling rank)	Sternalski et al. 2012a
Western Marsh-Harrier (*Circus aeruginosus*)	Cere/tarsus	Yes (carotenoid supplementation)	Yellowness[1]	Body condition/ T-cell-mediated immunity	None/positive	Sternalski et al. 2012b
Brown Falcon (*Falco berigora*)	Cere	No	Yellowness[2]	None	N/A	McDonald 2003

[1] In these studies, the authors used colorimetric charts, ranging from yellow to orange within a scale from 1 to 6.

[2] Yellowness was assessed assigning a value between 0 and 3 based on the degree of yellow present in the cere and orbital ring.

tissues (Trams 1969, McGraw and Parker 2006). Cholesterol is a main lipoprotein and carotenoids are mobilized through the blood stream bound to cholesterol molecules (McGraw and Parker 2006). When individuals are under physiological constraints, such as a food shortage or a pathogen-challenge, carotenoids are mobilized and used for self-maintenance rather than pigmentation (Blas et al. 2006, Martínez-Padilla et al. 2010, Sternalski et al. 2012a, 2012b). The coloration of carotenoid-pigmented features should therefore be particularly sensitive to stressful conditions (Mougeot et al. 2010b). Food shortage and carotenoid limitation via diet acquisition are typically associated with elevated stress hormone levels (Romero 2004), and elevated stress hormone levels are associated with reduced immunocompetence (Buchanan 2000, Loiseau et al. 2008), implying a greater need for using carotenoids for self-maintenance rather than for trait pigmentation (Blount et al. 2003). This is particularly relevant during growth in altricial species, including most raptors, as nestlings depend on the food provided by parents and have to compete with their siblings (Sternalski et al. 2012a, 2012b).

Reversed sexual size dimorphism is common in most raptor species, where females are frequently bigger and heavier than males (Newton 1979). This has been shown to have important consequences for sibling competition during growth (Oddie 2000, Fargallo et al. 2002, 2003) and during the post-fledging period (Vergara and Fargallo 2008, Vergara et al. 2010) that may drive adaptive strategies of differential parental investment among sexes to mitigate such competition (Blanco et al. 2003a, 2003b, Martínez-Padilla 2006). However, understanding which sex nestling is favored under stressful conditions during growth remains difficult. Females, which are larger than males, might be better competitors for food than their siblings (Arroyo 2002, Fargallo et al. 2002). Alternatively, females may be also more likely to suffer from food shortages, because of their higher energetic requirements during growth (Martínez-Padilla and Viñuela 2011).

The goal of our study was to investigate how corticosterone levels in feathers, as an index to stress during growth, are associated with condition (body mass and circulating cholesterol levels), circulating carotenoid levels, and the coloration of carotenoid-pigmented features (the yellow-orange coloration of the cere and tarsi) in Common Buzzard (*Buteo buteo*) nestlings. We predicted that levels of carotenoid-based signaling and feather corticosterone levels would be negatively related, and nestlings in poorer condition would have greater feather corticosterone levels. Under stressful conditions, nestlings might be more carotenoid-limited or less able to allocate carotenoids to bare parts, because they would have used these carotenoids for other physiological needs. We further investigated possible differences between sexes, especially relative to the mediating effect of corticosterone levels on the relationship between coloration and circulating carotenoid levels.

METHODS

Nest Monitoring. Our study areas included the MacRobert estate, Forest of Birse, Birse estate and Glen Tanar estate (Aboyne, Aberdeenshire, Scotland), 2005 and 2006. We searched for occupied territories in April–May by means of behavioral observations made from high points during early morning or late evening. Common Buzzards that were calling, carrying branches or prey, or performing repeated straight flights toward a specific area of the forest were considered potential nesting pairs. We searched for nest sites in each nesting area by looking for nest in the trees around the edges of the forests. Nests were initially monitored from a distance, with binoculars or a spotting scope. Nests were visited before the nestlings were old enough to leave the nest, at the average age of 34 d old (range = ±6 d). Wing length was used to estimate nestlings' age (based on J. Martinez-Padilla unpubl. data).

We climbed to accessible nests using standard tree-climbing techniques. During nest visits, we measured wing length (with a ruler, to the nearest 1 mm) and mass (with a pesola balance, to the nearest 2 g) for each nestling. Rank order within the brood was determined by body mass and nestlings were classified as older (heavier), intermediate, and younger (lighter). When there were only two nestlings, the intermediate category was not included. We took a 2-ml blood sample from the brachial vein and digital photographs of the cere and tarsi (see below). Finally, we collected a growing feather (the first secondary from the left wing) for assaying corticosterone levels. The sex of nestlings was determined from blood samples using molecular methods as described in Fridolfsson and Ellegren (1999).

Color Assessment. We measured the carotenoid-based coloration of legs (tarsi) and cere of bills using digital photographs (Martínez-Padilla et al. 2007, 2011). High-resolution (2272 × 1704 pixels) lateral pictures of these two traits were taken from a standard distance (40 cm) and using the flash incorporated in the digital camera (Nikon Coolpix

950ZS). For each photograph, we placed the same reference card next to legs or cere, which provided a reference to standardize coloration and illumination of all photographs. To avoid flash reflections on the photographed reference or trait, the photographs were taken at an angle of approximately 45°. We analyzed digital images using Adobe CS5. We measured Hue (H, in degrees), Saturation (S) and Brightness (B) for the cere, leg and reference. Higher hue values were associated with paler yellow and lower values with more orange-yellow coloration. Specifically, we selected a rectangle within the color reference for each photograph to obtain H, S, and B. The same color reference was used for all photographs. In order to assess cere and leg coloration, we used the tool laze in CS5 with a tolerance rate of 35% and selected a homogeneous area within the trait (cere or leg), where we obtained H, S, and B for each trait along the H, S, and B of the color reference. In our color assessments, we did not consider UV reflectance, but we are confident that our measuring degrees of yellow coloration with a digital photograph was a reliable proxy to assess coloration of carotenoid-pigmented bare parts (Mougeot et al. 2007).

Circulating Carotenoid and Cholesterol Levels. Blood was kept refrigerated until centrifugation, within 2–3 hr of collection. Plasma samples were obtained after centrifuging blood for 10 min at 10 000 rpm and were stored at −80°C until analyzed. Carotenoids were quantified by diluting 60 µl of plasma in acetone (1:10). The mixture was vortexed and centrifuged at 10 000 rpm for 10 min. We examined the supernatant in a Shimadzu UV-1603 spectrophotometer and determined the optical density at 446 nm, the wavelength of maximal absorbance for lutein (Mínguez-Mosquera 1993). Lutein is the main carotenoid in raptors, and is responsible for the yellow cere and tarsi coloration (e.g., Bortolotti et al. 2003, Sternalski et al. 2010). Carotenoid concentrations were calculated using the published extinction coefficient for this carotenoid pigment in acetone (Mínguez-Mosquera 1993).

Plasma cholesterol levels were analyzed by the oxidase-peroxidase method, with commercial kits purchased from BioSystems S.A. and an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain; Pérez-Rodríguez et al. 2007).

Feather Corticosterone Assays. We extracted corticosterone from feathers using a methanol-based extraction technique (Bortolotti et al. 2008). The feather less the calamus was first minced into pieces of <5 mm^2 with scissors. We then added 10 ml of methanol (HPLC grade, VWR International, Mississauga, Ontario, Canada) and placed the samples in a sonicating water bath at room temperature for 30 min, followed by incubation at 50°C overnight in a shaking water bath. The methanol was then separated from feather material by vacuum filtration, using a plug of synthetic polyester fiber in the filtration funnel. The feather remnants, original sample vial, and filtration material were washed twice with approximately 2.5 ml of additional methanol; the washes were added to the original methanol extract. The methanol extract was placed in a 50°C water bath and subsequently evaporated in a fume hood. Evaporation of the samples was completed within a few hours and the extract residues were reconstituted in a small volume of the phosphate buffer system (PBS; 0.05M, pH 7.6) used in the corticosterone (CORT) radioimmunoassay (Blas et al. 2005). The filtration step was generally found to be sufficient to remove feather particulates but further particulate material could be removed, if needed, by centrifugation of the PBS-reconstituted samples. Reconstituted samples were frozen at −20°C until analyzed for CORT. We assessed the efficiency of the methanol extraction by including feather samples spiked with a small amount (approximately 4000 DPM) of ^{3}H-corticosterone in each extraction. More than 90% of the radioactivity was recoverable in the reconstituted samples. Data values are expressed as pg CORT per mm of feather, which gives a valid estimate of CORT per unit time of feather growth (Bortolotti et al. 2008). CORT assays were performed at the University of Saskatchewan, Canada.

Statistical Analyses. We used General Linear Mixed Models (GLMM) in SAS 9.2. Nestling mass, corticosterone levels, carotenoid levels, cholesterol levels, and coloration parameters (H, S, and B) were considered the dependent variables. Nestling sex was considered a fixed factor. To explore the mediating effect of nestling sex on coloration and stress levels, we considered the interaction between corticosterone levels and nestling sex, with color variables of cere and leg as response variables. Rank order was considered a factor in all models and coded as 1 (heavier), 2 (intermediate) or 3 (lighter). Nest identity (i.e., brood) was included as random factor in all models, to account for the nonindependence of nestling data from the same brood. When H, S, or B were used as dependent variables, the corresponding value of H, S, or B of the reference for each photograph was included as a covariate in the model. Sample size may differ among

Table 2. Mean (±SEM) values for all the study variables in male and female Common Buzzard nestlings separately, and for nestlings of both sexes combined. Sample sizes refer to the number of individuals sampled.

	MALES		FEMALES		ALL	
PARAMETER	*n*	AVERAGE ± SE	*n*	AVERAGE ± SE	*n*	AVERAGE ± SE
Nestling mass[1]	19	807.63 ± 28.50	17	914.4 ± 25.91	36	861.02 ± 21.13
Corticosterone levels[2]	14	2.89 ± 0.44	17	2.91 ± 0.29	31	2.90 ± 0.25
Carotenoid levels[3]	17	11.44 ± 1.01	15	9.56 ± 0.49	32	10.50 ± 0.60
Cholesterol levels[4]	16	252.06 ± 17.13	15	265.60 ± 15.60	31	258.61 ± 11.50
Hue of the cere[5]	16	61.69 ± 1.76	15	61.93 ± 1.51	31	61.81 ± 1.50
Saturation of the cere	16	59.00 ± 3.17	15	59.2 ± 3.45	31	59.1 ± 2.30
Brightness of the cere	16	63.44 ± 1.83	15	65.93 ± 2.66	31	64.69 ± 1.59
Hue of the leg[5]	16	53.00 ± 1.70	14	50.5 ± 1.17	30	51.75 ± 1.07
Saturation of the leg	16	42.81 ± 2.73	14	48.86 ± 2.98	30	45.83 ± 2.06
Brightness of the leg	16	61.25 ± 1.80	14	66.64 ± 2.20	30	63.95 ± 1.47

[1] In g.
[2] In pg/mm of feather.
[3] In μg/ml.
[4] In mg/dL.
[5] In degrees.

analyses depending on study variables because of fieldwork or sampling limitations (not all parameters could be measured for each nestling). Tests were two-tailed and data are expressed as mean ± SEM.

RESULTS

We sampled 36 nestlings from 15 nests. Neither circulating carotenoid levels, corticosterone levels (CORT), nor any index of coloration of cere or tarsi significantly differed between sexes (all $P > 0.217$, Table 2, 3). Only nestling mass differed between males and females ($F_{1,18} = 26.86$, $P < 0.001$), with females being heavier than males (Table 2). Nestling mass was negatively correlated with corticosterone levels ($F_{1,26} = 6.27$, $P = 0.026$; $F_{1,18} = 18.93$, $P < 0.001$ when including sex as a factor: slope: -47.08 ± 16.11, Fig. 1a). Circulating carotenoid levels were positively correlated with feather corticosterone levels ($F_{1,18} = 4.79$, $P = 0.047$; slope: 0.71 ± 0.63, Fig. 1b). These associations did not differ between sexes (nonsignificant Sex × CORT interactions; both $P > 0.373$) or rank order within the brood (all $P > 0.440$).

Hue, saturation, and brightness values of the cere ranged from 47°–77°, 31–88, and 51–87, respectively. Hue, saturation, and brightness of the leg ranged from 43°–71°, 22–67, and 50–83, respectively. The relationship between the hue of cere and corticosterone levels was sex-dependent (Table 3). Specifically, cere hue was negatively correlated with CORT in females ($F_{1,11} = 20.93$, $P < 0.001$; slope: -4.043 ± 0.884; Fig. 2a), but not in males ($F_{1,10} = 0.76$, $P = 0.403$; slope: 0.687 ± 0.787; Fig. 2a). Similarly, the relationship between the hue of tarsi and corticosterone levels tended to differ between sexes (Table 3). There was a marginal (but nonsignificant) negative association between leg hue and corticosterone levels in females ($F_{1,9} = 3.31$, $P = 0.102$; slope: -2.39 ± 1.32, Fig. 2b), but no association in males ($F_{1,11} = 1.14$, $P = 0.310$; slope: 0.86 ± 0.81; Fig. 2b).

Plasma cholesterol levels varied with corticosterone levels, but this association was also sex-dependent (significant CORT × sex interaction: $F_{1,11} = 15.28$, $P = 0.002$, Fig. 3). In females, the relationship was significant and negative ($F_{1,9} = 20.09$, $P = 0.020$; slope: -40.89 ± 9.12; Fig. 3), whereas there was no association in males ($F_{1,11} = 2.83$, $P = 0.234$; slope: 14.20 ± 8.44; Fig. 3). There were no significant associations between cholesterol levels and any of the other study variables (all $P > 0.081$).

The saturation or brightness of both cere and tarsi were not related to carotenoid levels, irrespective of nestling sex (Table 3).

DISCUSSION

We predicted a negative association between carotenoid-based coloration and feather corticosterone levels in buzzard nestlings. This type of association was only partly supported by our data, with negative associations between cere hue and tarsi hue apparent in females but not in males. We also found that feather corticosterone levels were nega-

Table 3. Results of the GLMMs testing for associations between carotenoid-based coloration and carotenoids (first three rows) and corticosterone (last three rows). Coloration parameters included the hue, saturation, and brightness of legs and cere. Nestling sex was included in the models to test for sex-specific relationships. All models also included the color values for the reference, in order to standardize color measurement between images. Nest was included as random variable in all models. *P* values in bold highlight significant or marginally significant effects. The relationship between corticosterone levels and saturation and brightness of the leg had $P < 0.05$; although significant, this was due the effect of the interaction (Cort. × Sex). When the interaction was removed, there was no relationship between corticosterone and saturation ($F_{1,23} = 3.17$, $P = 0.090$) or brightness ($F_{1,23} = 1.00$, $P = 0.327$) of the leg.

MODEL	CERE									LEG								
	HUE			SATURATION			BRIGHTNESS			HUE			SATURATION			BRIGHTNESS		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Carotenoids	1,11	0.59	*0.460*	1,11	0.11	*0.748*	1,11	0.00	*0.979*	1,9	0.28	*0.607*	1,9	1.38	*0.270*	1,9	1.28	*0.288*
Sex	1,11	1.71	*0.217*	1,11	0.00	*0.974*	1,11	0.03	*0.875*	1,9	0.34	*0.576*	1,9	0.65	*0.441*	1,9	0.60	*0.458*
Carotenoid × Sex	1,11	1.38	*0.265*	1,11	0.01	*0.940*	1,11	0.03	*0.865*	1,9	0.54	*0.539*	1,9	0.23	*0.641*	1,9	0.51	*0.494*
Corticosterone	1,11	4.46	*0.058*	1,11	0.30	*0.594*	1,11	0.08	*0.780*	1,9	1.23	*0.297*	1,9	7.42	*0.023*	1,9	6.90	*0.028*
Sex	1,11	6.45	*0.028*	1,11	0.71	*0.418*	1,11	0.14	*0.713*	1,9	3.43	*0.970*	1,9	0.93	*0.359*	1,9	0.07	*0.797*
Cort. × Sex	1,11	7.01	***0.023***	1,11	1.06	*0.325*	1,11	0.37	*0.556*	1,9	5.06	***0.051***	1,9	3.46	*0.096*	1,9	0.04	*0.849*

tively related to nestling mass and positively related to circulating carotenoid levels. Interestingly, carotenoid levels were not associated with cere or leg coloration, as expected for carotenoid-pigmented traits.

We found a negative relationship between nestling mass and corticosterone levels. In our study, nestling mass covaried with age, and a previous study on American Kestrels showed that corticosterone levels increase with age (Love et al. 2003). Increases in corticosterone may increase appetite and begging behaviors, thereby increasing the probability of being better fed (Loiseau et al. 2008), and perhaps increasing the probability of fledging earlier (Belthoff and Dufty 1998). Considering that nestling mass is a proxy of age in our study, our results suggest the opposite. Our results agree with the premise that stress may lead to a mobilization of resources, with the result that nestlings with higher corticosterone levels are not well fed and are lighter. This has been previously shown by means of condition loss, considering nestling mass loss as a proxy of body condition (Cote et al. 2006, Loiseau et al. 2008). Individuals growing in stressful environmental conditions, such as with reduced food availability, may experience chronic stress and more frequent food shortages during growth, which may explain a negative relationship between nestling mass and corticosterone levels. Stressful conditions may trigger the mobilization of lipids where carotenoids are stored, and therefore their release into the blood stream (McGraw and Parker 2006, McGraw et al. 2011), which may explain the observed positive relationship between circulating carotenoids and corticosterone levels.

The lack of relationship between carotenoid levels and cere or tarsi coloration was interesting. There are several, non-exclusive, possible explanations. First, stressful conditions may induce a mobilization and rapid use of carotenoids for physiological functions other than those leading to increased coloration. Carotenoids are immunostimulants, and may have important antioxidant functions, and are therefore essential to maintaining key physiological processes (Pérez-Rodríguez 2009). Under stressful conditions, individuals may have different allocation priorities for carotenoids (Mougeot et al. 2009, Martínez-Padilla et al. 2010). Long-term exposure to elevated levels of corticosterone may lead to immune-suppression (Romero 2004, Loiseau et al. 2008) and increased oxidative stress (Costantini et al. 2011), prompting a use of available carotenoids

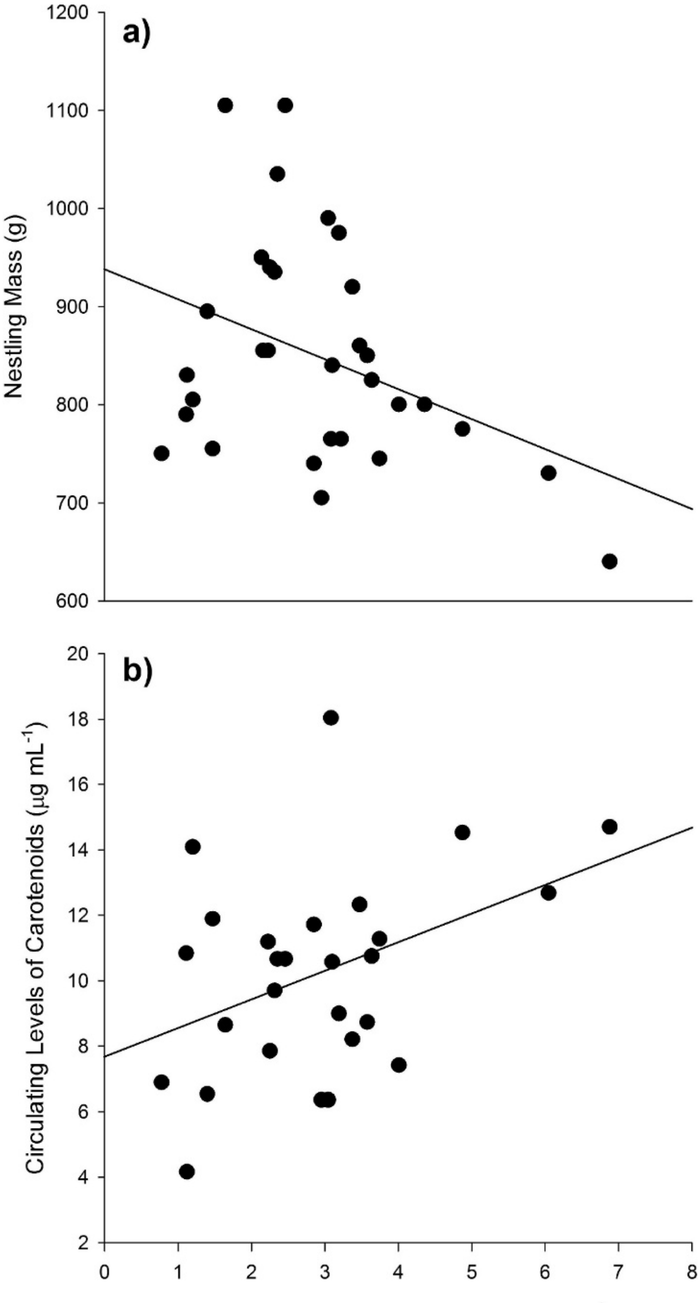

Figure 1. Associations between (a) nestling mass and feather corticosterone levels, and (b) circulating carotenoid levels and feather corticosterone levels (data from both sexes combined).

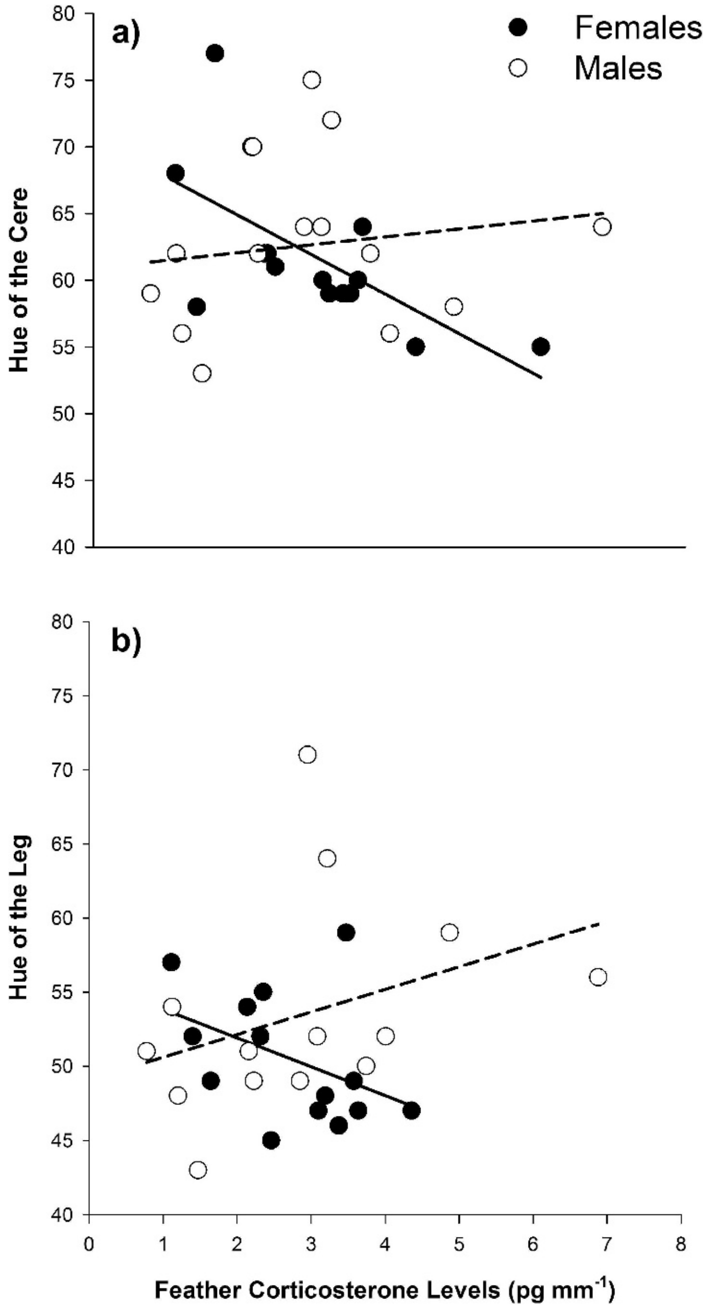

Figure 2. Sex-specific associations between (a) cere hue or (b) leg hue and feather corticosterone levels in Common Buzzard nestlings. Note that higher hue values are related to paler yellow (see Methods for further details).

as immunostimulants or as antioxidant protection. Individuals could use carotenoids to boost their immune systems and compensate for stress-induced immunosuppression (McGraw and Ardia 2007), although this relationship needs further clarification (Pérez-Rodríguez 2009). Therefore, a lack of association between carotenoid-based coloration and circulating carotenoid levels may arise when caroten-

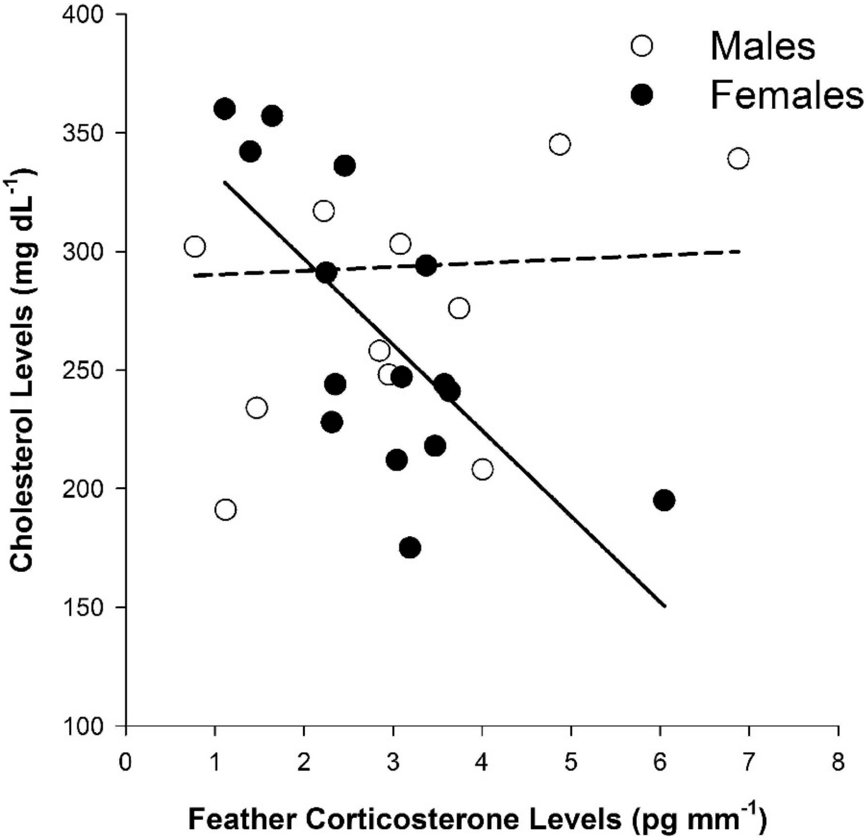

Figure 3. Sex-specific association between cholesterol levels and feather corticosterone levels in buzzard nestlings.

oids are not directly allocated to the coloration of integuments (Peters et al. 2008). We suggest that corticosterone may have a crucial mediating role, modulating phenotypic expression under chronic stress exposure (Bortolotti et al. 2009b). In red grouse, changes in carotenoid and coloration levels closely mirrored changes in corticosterone levels deposited in feathers (Mougeot et al. 2010b). In Common Buzzard nestlings, our results suggested that developing in a more stressful environment is associated with a reduced carotenoid availability for physiological functions. Alternatively, it is possible that carotenoids can be allocated to bare parts for coloration, but that the time elapsed between mobilization and deposition of the carotenoids may be longer than the time needed to detect the physiological effects of starvation. Similarly, it is also possible that corticosterone levels obtained from feathers do not accurately reflect the physiological state of the nestling at the time we assessed coloration and carotenoid levels.

The expected negative association between feather corticosterone levels and carotenoid-based coloration (Mougeot et al. 2010a) was found for both cere and leg hue, but only in female nestlings. Therefore, our results only partially agreed with prior studies, because this relationship was sex-dependent. Stressful conditions during growth of buzzard nestlings may have different physiological effects on males and females. Our findings suggest that coloration of cere and legs might reflect such stressful conditions in females only. Parasites have been suggested as a key component of the complex interrelationships between corticosterone levels, carotenoid levels, and ornament expression (Bortolotti et al. 2009b, Mougeot et al. 2010a, 2010b), so differences in parasite infection levels between males and females may explain the observed sex-specific associations. Indeed, some parasites can be more abundant in female than in male nestlings in the Common Buzzards in eastern Westphalia in Germany (Chakarov et al. 2008). We recommend that future studies investigate parasite burdens of male and female nestlings relative to stress levels and coloration.

Sexual size dimorphism contributes to sex-dependent competitive abilities in broods with marked brood size hierarchy, particularly in raptors that are characterized by a reversed sexual size dimorphism (Newton 1979). Female nestlings are larger and heavier than males, and may have greater energetic requirements, which may make females more sensitive to stressful conditions, as previously hypothesized for some raptor species (Martínez-Padilla and Fargallo 2007, Martínez-Padilla and Viñuela 2011), and body condition may alter the relationship between coloration and corticosterone levels (Loiseau et al. 2008). However, females, as the bigger sex, may have the advantage for getting food provided by parents (Arroyo 2002, Fargallo et al. 2002). Counterintuitively, the negative relationship between corticosterone levels and hue of the cere and leg in females means that higher stress levels are associated with more colored bare parts (lower hue values); this contrasted our expectation that more stressed birds would show paler coloration of their cere and legs. From a mechanistic point of view, cholesterol plays a significant role in carotenoid mobilization and transport through blood stream in birds (McGraw et al. 2006, McGraw and Parker 2006). We found that cholesterol levels were negatively associated with feather corticosterone levels in females only. It is possible that more-stressed females have lower cholesterol levels, possibly due to allocation of carotenoids (bound to cholesterol) for cere and leg coloration. The reason of this sex-dependent mobilization or use of cholesterol is uncertain, but it supports the idea that females respond in a different manner than males under stressful situations.

In conclusion, our results suggested that stress hormones, and, in particular, corticosterone, the main avian glucocorticoid, may influence how carotenoids are allocated and used by raptor nestlings for trait coloration or other needs. Our study revealed a sex-specific mediating role of this stress hormone in raptor nestlings. Measuring corticosterone deposited in feathers has potential for revealing how nestlings respond to a wide range of stressors experienced during growth.

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